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CLAIM FOR PRIORITY UNDER 35 U.S.C. § 119

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Sir:

A claim for priority is hereby made under the provisions of 35 U.S.C. § 119 for the above-identified national stage patent application based upon Australian Patent Application No. PQ0023 filed April 28, 1999.

Respectfully submitted,

Date: January 11, 2002

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I, ANNA MAIJA MADL, ACTING TEAM LEADER EXAMINATION SUPPORT & SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 0023 for a patent by THE AUSTRALIAN NATIONAL UNIVERSITY filed on 28 April 1999.

WITNESS my hand this
Twelfth day of May 2000

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The Australian National University

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Model Membrane Systems"

The invention is described in the following statement:

MODEL MEMBRANE SYSTEMS

The present invention relates generally to anchoring molecules to model and biological membrane systems, and to the use of anchored molecules in assays of inter molecular interactions and to modify biological responses. In one form, the present invention provides the basis of a novel method for the screening of drugs and other agents which affect intermolecular interactions. In another form, the invention provides a means of modifying the properties of biological and/or synthetic membranes and liposomes for the purpose of altering immunity when used as vaccines, or for the targeting of drugs and other agents to specific cells or tissues when administered *in vivo* for either therapeutic purposes or for modifying physiological responses or function.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

In biological systems such as cells, bacteria or viruses, surface biomolecules or receptors often exist as molecular structures consisting of two or more molecular components called subunits; these subunits may be identical, or molecularly distinct. The binding of natural ligand molecule(s) to receptor subunits may induce noncovalent aggregation or oligomerization of these receptor components. The oligomerization event is often an essential part of the mechanism by which the receptor can transduce transmembrane signals for triggering the induction of biological responses by the ligand molecule(s). In addition, the ability of certain receptors, or components thereof, to aggregate spontaneously may affect their ability to interact with ligands. Ligand molecules may be growth factors, cytokines, hormones, proteins, glycoproteins, polysaccharides, or any surface exposed or subcellular component of a cell, viral or subviral particle, or other infectious agent, which can bind to the receptor.

By virtue of their ability to undergo multimeric interactions, oligomerized receptors often have the potential to interact stably with ligands of low binding affinity. However, for many receptors the affinity of self-association or interaction with ligands is not high enough to allow detection using conventional binding techniques, which often require covalent labelling, solubilization with detergents, or immobilization of the receptor or ligand onto the solid sensing surface of an optical biosensor. These methods are suited to the study of relatively high affinity interactions and they generally rely on the ability of the molecules to interact in solution or to maintain stable interaction after cell disruption. Since the effective receptor/ligand concentration in solution is reduced compared to that on the two-dimensional surface of a cell (where molecules can oligomerize or cluster and interact stably with other molecules through multimeric interactions) these methods are limited in their ability to detect interactions of low affinity.

Atomic force microscopy (also known as scanning probe microscopy) allows three-dimensional imaging and measurement of structures ranging in size from atomic dimensions to microns, and is revolutionary in its ability to resolve structures never seen before (1). The development of optical biosensors has permitted the monitoring of the interaction between macromolecules in real time (2). To date, both of these techniques generally have been used with the receptor or the ligand molecule covalently attached to or immobilised onto a solid surface (1-4).

Recently a technique has been described in which the linkage of a recombinant hexa-histidine-tagged protein with nitrilotriacetic acid (NTA) is used to reversibly immobilize a hexa-histidine-tagged protein onto the solid sensing surface of a BIAcore surface plasmon resonance biosensor (5). The formation of a hybrid octadecanethiol/phospholipid membrane on the BIAcore sensing surface also has been described (6), enabling analysis of the binding of streptavidin to biotinylated phosphatidylethanolamine in the bilayer. In addition, the immobilisation of histidine-tagged biomolecules to bilayer membranes via the chelator lipids NTA-dioctadecylamine has been demonstrated byepifluorescence microscopy and film balance techniques (7-8). These prior art techniques do not permit an analysis of an interaction of receptor domains under conditions that mimic a cellular or subcellular membrane that allows lateral mobility of the receptors, and that allows for the possibility of studying receptor oligomerization and multimeric interactions with ligands. Furthermore, these prior art techniques

do not permit screening for drugs or other agents that may influence such molecular interactions.

In one embodiment, the present invention contemplates a method of anchoring the extramembranous or transmembrane domains of receptors to overcome one or more of the foregoing shortcomings of the prior art.

In another embodiment, the subject invention provides a method of assaying interactions between membrane anchored molecules and between anchored molecules and molecules capable of interacting therewith. More particularly, the present invention is useful to study interactions between extramembranous or transmembrane domains of receptors, and between receptor domains and a ligand(s), by anchoring the receptor domain(s) onto a fluid membrane system. Receptor domains may also be composed of proteins, glycoproteins or proteoglycans, oligosaccharides, or fragments or functional equivalents thereof.

An important application of the present invention, therefore, is in providing the technology for the screening of agents or drugs which affect either induced or spontaneous receptor aggregation and, hence, which influence receptor function. The present invention may also be used in the screening for drugs which disrupt pre-existing aggregates of receptor subunits, such as the normally associated a and b subunits of the MHC class II molecules described below.

The present invention is useful to monitor the spontaneous or ligand induced aggregation/ disaggregation processes of receptors or protein fragments which are not normally membrane associated, once they are engineered to have a hexa-histidine tag and anchored to the membrane as described below for the cell surface derived molecules CD4 and MHC class II. Alternatively, a receptor domain or related protein fragment can be engineered to contain a suitably located strand which spontaneously inserts into the outermost leaflet of the membrane bilayer. The application of this system includes drug screening and surveying for new ligand species or biochemical agents (eg. enzymes) which induce modifications that affect aggregation processes.

Thus, the present invention is directed to a method of anchoring receptor domains (albeit extramembranous or transmembrane) onto a membrane such that said receptor domains are

capable of lateral movement to facilitate interactions with biomolecules in the membrane.

Accordingly, the present invention provides a method of anchoring receptor domains onto a planar supported membrane comprising amphiphilic molecules arranged in a bilayer, wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group such that some of the metal chelating groups are oriented toward the outside surface of said membrane; which method comprises the step of interacting a receptor domain which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach to said membrane via the outwardly facing metal chelating residues of said membrane, such that the receptor domains are capable of lateral movement.

A preferred metal chelating group for use in the present invention is nitrilotriacetic acid (NTA).

The membrane may be formed from a suspension of micelles (e.g. liposomes) from the amphiphilic molecules, wherein a proportion of the amphiphilic molecules have been modified by covalent attachment of a metal chelating group.

Accordingly, another aspect of the invention provides a method of anchoring receptor domains onto a membrane such that the receptor domains are capable of lateral movement, said method comprising of:

i) forming a suspension of micelles (e.g. liposomes) from amphiphilic molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group

ii) interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a bilayer membrane to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

iii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach via the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane.

The amphiphilic molecules are normally surfactant molecules having a hydrophilic "head" portion and one or more hydrophobic "tails". Surfactants may be any of the known types, i.e. cationic (e.g. quaternary ammonium salts), anionic (e.g. organosulfonate salts), zwitterionic (e.g. the phospholipids: phosphatidylcholines and phosphatidylethanolamines), membrane spanning lipid, or non-ionic (e.g. polyether materials).

The membrane may be comprised of more than one type of amphiphilic molecule. In a preferred embodiment, the membrane is comprised of a first phospholipid and a second phospholipid.

Thus, in a preferred form, the present invention contemplates a method of anchoring receptor domains onto a lipid bilayer such that said receptor domains are capable of lateral movement to facilitate interactions with biomolecules in the lipid bilayer, said method comprising of:

i) forming a suspension of micelles (e.g. liposomes) from a first phospholipid and a second phospholipid wherein the second phospholipid has been modified by a covalent attachment of a nitrilotriacetic acid (NTA).

ii) interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a phospholipid bilayer membrane to form in which some NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

iii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for

said polypeptide tag to attach via the NTA-metal chelating linkage to the outwardly-facing NTA residues of said membrane.

The method of this aspect of the present invention is particularly useful for anchoring extramembranous or transmembrane receptor domains.

In a further preferred form, the first phospholipid is phosphatidylcholine (PC) and the second lipid is phosphatidyl-ethanolamine-NTA (PE-NTA) and the ratio of PC:PE-NTA (w/v) is about 10:1. However, the first phospholipid can be any phospholipid or hydrocarbon capable of forming a lipid bilayer; and the second phospholipid can be any lipid with a metal chelating headgroup which can be used to anchor receptor domains using a suitably engineered tag on the domain. In addition, the ratio of the first to the second phospholipid can be varied depending on the desired density of receptor domain molecules to be achieved on the bilayer membrane.

In one form of the present invention, the support is the glass-like sensing surface of an optical biosensor cuvette or a mica surface suitable for analysis by atomic force microscopy. Preferably, the support is a planar glass or mica surface, but could be any surface which can form a substrate for a supported planar lipid bilayer membrane (6, 9, 10).

In another form, the surface may be a layer of glass, gold or any material which can be reacted with a solution of a hydrocarbon-containing compound (e.g. octadecyltrichlorosilane and octadecanethiol), capable of attaching hydrocarbon chains to said surface (6, 11), such that chains of hydrocarbon are oriented away from the surface of said support to form a lipid monolayer or a hydrophobic surface which is capable of reacting with a suspension of phospholipid micelles (e.g. liposomes) to form a planar bilayer membrane (6, 9, 10). In these forms the NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane. Such surfaces may be the sensing surface of optical biosensors that are capable of monitoring the interaction of receptor domains anchored on the membrane with ligand molecules in solution.

Preferably, the polypeptide tag comprises a sequence of at least six amino acid residues such as

a hexa-histidine molecule, but can be any sequence of amino acids that can bind strongly through the formation of a complex with the metal chelating component of a lipid containing a metal chelating group such as NTA. In one application of the subject invention the molecule is a transcription factor molecule. In another form of the instant invention, the molecule is a receptor. More particularly, the receptor may be any cell surface receptor such as the human cell surface molecule CD4 or human MHC class II molecule, or domains of such receptors.

Accordingly, in another aspect, the present invention provides a method of assaying interactions between a receptor and a ligand, comprising the steps of:

a) anchoring a receptor to a membrane by:

i) forming a suspension of micelles (e.g. liposomes) from amphiphilic molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group

ii) interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a bilayer membrane to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

iii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach via the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane.

b) allowing said receptor molecules to interact and/or oligomerize on the membrane; and

(c) contacting said anchored receptor with an effective concentration of ligand for a time and under conditions sufficient for binding to occur and detecting said binding.

In one embodiment, the present invention contemplates a method of assaying interactions between a receptor and a ligand comprising the steps of:

(a) anchoring a receptor to a lipid bilayer by

(i) forming a suspension of vesicles from a first phospholipid and a second phospholipid wherein said second phospholipid has been modified by covalent attachment of a metal chelating group such as nitrilotriacetic acid (NTA);

(ii) interacting said micelle (e.g. liposome) suspension with an appropriate planar support such as glass or mica for a time and under conditions sufficient to allow a hydrocarbon phospholipid bilayer membrane to form on the support in which some of the NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane;

(iii) interacting the receptor domain to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach via the NTA-chelating linkage to the outwardly-facing NTA residues of said membrane;

(b) allowing said receptor molecules to interact and/or oligomerize on the membrane; and

(c) contacting said anchored receptor with an effective concentration of ligand for a time and under conditions sufficient for binding to occur and detecting said binding.

In the case where the solid support (e.g. gold) may not be suitable for bilayer formation directly from the vesicle suspension, steps (i) to (iii) above are preceded by a step to form a monolayer or hydrophobic surface on the solid support. This is achieved by reacting the solid support with a solution of a hydrocarbon-containing compound capable of attaching hydrocarbon chains to its surface, such that chains of hydrocarbon are oriented away from the surface of said support to form a lipid monolayer or a hydrophobic surface. The surface is then reacted with a suspension of phospholipid amphiphilic molecule micelles (e.g. liposomes), preferably micelles (e.g. liposomes) [as in steps (i)-(ii) above] for a time and under the conditions necessary to form

a bilayer membrane. The metal chelating, preferably NTA residues attached to the amphiphilic molecules such as a second phospholipid will be oriented toward the outside surface of said membrane, and may be used to anchor molecules with a hexa-histidine tag [as described in (iii) above]. Such surfaces may be the sensing surface of optical biosensors that are capable of monitoring the interaction of receptor domains anchored on the membrane with ligand molecules in solution.

Preferably, the oligomerization or self-association of the receptor and binding to the ligand is detected by atomic force microscopy or by an optical biosensor technique.

The present invention thus provides for the anchoring of receptor domains, proteins, glycoproteins and polysaccharides, onto lipid bilayers formed on mica, glass, gold or any other appropriate surface, that enable the molecules to diffuse laterally and interact. This technology is ideal in a preferred embodiment for studying the interaction between receptor domains and between receptor domains and ligands in a membrane system using optical biosensor and atomic force microscope techniques.

Receptor domains can be engineered to have a hexa-histidine COOH-tail, or NH2-tail using standard recombinant DNA techniques. A hexa-histidine tag also may be covalently attached to polysaccharides and other molecules by chemical means.

Optical biosensors used in connection with this invention measure the change in refractive index due to the binding of a soluble macromolecule with a molecule immobilized onto the solid sensing surface and can be used to measure the binding affinity of the interacting molecules. The IAsys biosensor employs a sample cuvette which has a resonance mirror as the sensor surface. The glass-like sensing surface of the IAsys biosensor is ideal for reacting with a suspension of micelles (e.g. liposomes) to form a bilayer membrane on the sensing surface.

The present invention may also be used with a BIAcore surface plasmon resonance biosensor which utilises a gold sensing surface. The gold is first reacted with octadecanethiol to form an octadecane monolayer, and then reacted with a suspension of micelles (e.g. liposomes) to form

the bilayer.

Atomic force microscopes can be used for mapping molecular structures on the model bilayer membranes containing a limited number of molecular species. Such bilayers can be formed either directly on mica or glass surfaces by incubation with a suspension of micelles (e.g. liposomes). A hydrophobic monolayer also can be formed on glass or gold surface by treating with an appropriate reagent, and a hybrid bilayer membrane formed by reacting the surface of the monolayer with a suspension of micelles (e.g. liposomes) composed of the amphiphilic molecules, preferably an appropriate mixture of PC and PE-NTA to form the bilayer.

The anchoring of hexa-histidine tagged receptors onto model membrane systems of the present invention is also useful to anchor or graft receptors and other molecules onto liposomes or vesicles which through the specificity of the grafted molecules can target and deliver drugs, DNA/RNA or any therapeutic agent that can be encapsulated into the liposomes, to specific cell types or tissues when the liposomes are administered *in vivo*. Recombinant molecules also can be grafted onto synthetic liposomes or vesicles for the purpose of developing vaccines to produce specific biological or therapeutic effects.

According to this aspect of the present invention there is provided a method of grafting molecules onto liposomes said method comprising:

i) preparing a suspension of liposomes with chelator lipid incorporated;

ii) incubating the liposomes with a recombinant protein or target molecule bearing an appropriate metal affinity tag;

iii) if necessary, removing excess protein by washing, filtering or other washing means and suspending them in an appropriate solution.

In a preferred embodiment, the molecules may be anchored or grafted onto liposomes by the following method:

i) preparing a suspension of liposomes from an appropriate mixture of a chelator

lipid such as di-tetradecylamine nitrilotriacetic acid (di-C14-NTA) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) at a concentration of ~ 0.5 mM in aqueous solution such as PBS (phosphate buffered saline) containing a concentration of Ni2+ or Zn2+ approximately equal to that of the di-C14-NTA. The liposomes can be produced by sonicating the mixture for 5-10 mins at a temperature above the Tm. Alternatively, the liposomes can also be produced by dissolving the lipids in an ethanolic solution and then dispersing in acqeous buffer, or by extruding an acqeous suspension of the lipids through polycarbonate or similar filter of suitable pore size. Typically, the ratio of di-C14-NTA:POPC can be 5:1, but can be different;

ii) washing the liposomes by pelleting (by centrifuging at ~95,000 x g for 30 min at 4°C) and removing the supernatant, or by filtration techniques, and then suspending the liposomes in an appropriate volume of the buffering solution to facilitate incubation with the tagged protein(s);

iii) incubating the liposomes with a recombinant protein (e.g. human hexa-histidine-tagged VEGF, vascular endothelial growth factor), or a combination of different recombinant proteins, each bearing a hexa-histidine or other suitable metal affinity tag to allow it to be anchored onto the liposomes; and

iv) removing excess soluble or unincorporated protein by washing the liposomes as in step ii) above, then suspending them in PBS or other buffer solution suitable for administration *in vivo*.

Other combinations and different lipids can also be used in conjunction with the chelator lipid to give the liposomes specific properties. For example, the ganglioside GM1 or derivatives polyethylene glycol can be included in the mixture (in step i) above) to produce liposomes with "stealth" properties (12) to avoid them being taken up by macrophages or by the liver or spleen when used as vaccines *in vivo*. Also, step i) can be carried out in the presence of a drug, DNA

or other therapeutic agent for the purpose of encapsulating the material and permitting it, when administered *in vivo*, to be delivered to specific cells or tissues defined by the specificity of the grafted molecule(s). For example, liposomes with grafted VEGF (vascular endothelial growth factor) can be used to target angiogenic epithelium which is known to express the VEGF receptor and is required for tumor growth. Liposomes with grafted VEGF, therefore, can be used to deliver a cytotoxic drug or agent that can block the growth of new blood vessels needed for the growth of tumors.

Current methods of modifying the surfaces of cells to be used as vaccines for altering immunity to disease (e.g. the immune response to tumors - see below) generally require the transfection or genetic manipulation of the tumor cells, to induce them to express one or more specific protein(s) on their surface (13-15). For example, in both animal and human tumor models evidence suggests that the transfection of tumor cells with genes inducing them to express T cell costimulator molecules like B7-1 (CD80), B7-2 (CD86), CD40 and ICAM-1 on their surface, may be a useful approach to prepare the cells for use in vaccinations to enhance tumor immunity in the tumor bearing host (16-22). Unfortunately, in a clinical setting, such as in the treatment of cancer in humans, the transfection of tumor cells with such genes can be time consuming and inconvenient. Thus, the frequency of transfection is generally low, and successful transfection with multiple genes (to induce expression of multiple proteins on the tumor cell surface) can be difficult to achieve. Furthermore, transfection techniques, even when carried out by the use of seemingly harmless viral vectors, can be associated with risks to the patient owing to the difficulty in precisely controlling the expression of the gene or its integration into the genome.

The present invention further provides a more convenient and safe method of grafting costimulatory and other molecules directly onto the surfaces of cells (such as tumor cells) and other membranous structures (either biological or synthetic), that can be used as vaccines to enhance or modify immunity to tumors and other diseases in humans.

The method of anchoring receptors onto model membrane systems to assay for inter molecular interactions and drug screening can be used to anchor or "graft" molecules directly onto biological membranes (e.g. the membranes of cells or subcellular particles), once a chelator lipid

(e.g. di-C14-NTA) has been incorporated into the membranes, thereby providing a convenient way of grafting recombinant molecules possessing a hexa-histidine or other suitable metal affinity tag, directly onto the membrane surface.

In this aspect of the present invention there is provided a method of "pasting" recombinant molecules directly onto biological membranes said method comprising:

i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

ii) incubating a suspension of cells or biological membranous structures with a suspension of the chelator lipid;

iii) washing away excess or unincorporated lipid;

iv) incubating the membranous structures with a solution of recombinant protein or target molecule possessing an appropriate metal affinity tag; and

v) washing away excess or unbound soluble protein, and suspending the structures in a solution suitable for administration *in vivo*.

In a preferred embodiment the invention allows the grafting of molecules onto cells and other membranous structures using the following method:

i) washing a suspension of the cells or membranous structures with PBS or other aqueous buffer solution to remove excess soluble and/or loosely bound proteins. This can be carried out by pelleting the structures by appropriate centrifugation (e.g. 5 min at 200-500 x g for murine and human cells), and then resuspending them in PBS; depending on the structures, excess soluble or loosely bound proteins may be removed by filtering or other washing means;

ii) preparing a suspension of chelator lipid (e.g. di-C14-NTA, at a concentration of ~0.1 mM) in PBS containing an approximately equal concentration of either Zn2+ or Ni2+ by sonicating for 5-10 min an appropriate quantity of the lipid in the PBS solution. Other lipids or phospholipids (e.g. POPC) or other agents also can be included with the chelator lipid to promote the fusion and incorporation

of the liposomes into the membrane structures;

iii) incubating the cells or membranous structures with a suspension of the chelator lipid (e.g. 0.1 mM di-C14-NTA) in PBS for a suitable period of time and temperature (e.g. 30 min, at 37°C) to allow some of the lipid in the suspension to fuse and/or become incorporated into the structures. Note: the incubation conditions employed can be altered to suit the nature of the chelator lipid used and the particular membrane structure into which the lipid is to be incorporated; also, incubations or wash steps in buffer containing additives such as polyethylene glycol can be used to promote lipid fusion and incorporation;

iv) removing unincorporated lipid from the mixture by washing the membranous structures with PBS by pelleting and washing as in step i) above;

v) incubating the washed structures containing incorporated chelator lipid with a solution of a recombinant protein, or a solution of a mixture of different recombinant proteins, each containing a hexa-histidine or any other appropriate metal affinity tag; and

vi) washing the cells or structures with PBS (as in step i) above) to remove excess or unbound soluble recombinant protein.

A similar procedure can be used to graft tagged proteins to any cells, or subcellular membranous components. The structures so treated will contain a modified surface due to the grafted protein, and can be used in vaccinations to alter immunological responses *in vivo*. The structures when administered *in vivo* also can be used to target a particular cell type or tissue within the body thereby altering the function of these cells. For example, the grafting of tumor cells with molecules known to bind receptors on dendritic cells can be employed to direct the grafted tumor cells to the dendritic cells to enhance tumor antigen presentation and hence immunological responses against the tumor.

In this form, the present invention contemplates a method of anchoring or grafting recombinant receptors and other molecules directly onto biological membranes, such as the membrane of cells, and, hence, of modifying the properties of such membranes. In particular, the instant invention provides the basis of a convenient strategy for modifying the surfaces of cells (e.g. tumor cells), any cellular or subcellular membranous component, infectious agent or particle (e.g. bacteria), as well as any biological or synthetic membrane including synthetic vesicles or liposomes, into which the chelator lipid can be incorporated. In all these instances, the recombinant protein is grafted by the formation of a metal chelating linkage between a peptide tag on the protein, and the NTA headgroup on the chelator lipid incorporated into the membranous structure. The biological membrane being modified by the anchoring of any recombinant protein, glycoprotein and any other molecular structure possessing an appropriate tag, and designed to enhance immunity to diseases when used either as a vaccine, or as an agent to target delivery of these biological membranes to specific cells and tissues when administered *in vivo*.

Various aspects of the present invention will now be described, by way of example only, with reference to some specific examples and potential applications. In the ensuing description, reference is made to the accompanying drawings in which:

Figure 1 is a schematic diagram showing anchoring of a hexa-histidine-tagged protein to a model membrane formed on the glass sensing surface of an IAsys biosensor cuvette; and

Figures 2(a) and 2(b) are traces showing the change in refractive index as a function of time due to binding of molecules as monitored with an IAsys biosensor.

Figure 3 is a biosensor trace showing changes in refractive index due to additions made to an IAsys biosensor cuvette after reacting the sensing surface of the cuvette with a suspension of micelles (e.g. liposomes) to form a bilayer.

Figure 4 is an illustration of the way hexa-histidine-tagged molecules can be grafted onto the membranes of cells or other membranous structures.

Figure 5 is the fluorescence profile, as measured by fluorescence-activated cell sorting, of P815 cells grafted with biotinylated and hexa-histidine-tagged CD40 and B7.1 molecules and then stained with streptavidin-FITC.

Table 1 is the result of 3H-thymidine incorporation showing that grafted CD40 and B7.1 molecules can induce T cell proliferation in an allergeneic T cell proliferation assay .

As shown in Figure 1, to anchor hexa-histidine-tagged proteins onto a membrane for studying protein interactions with the IAsys biosensor, a lipid bilayer is first formed on the sensing surface of the biosensor cuvette using a known method which involves reacting the surface with a suspension of micelle (e.g. liposome)s made from 90% PC and 10% PE-NTA. The PE-NTA for these experiments was produced by first reacting PE with the bifunctional cross-linker bis(sulfosuccinimidyl)suberate, and then reacting the mixture with NTA; the NTA-PE product was purified by thin layer chromatography (and its identity confirmed by mass spectroscopy). The bilayer is then washed to remove excess micelle (e.g. liposome) suspension and equilibrated in Tris-buffered saline (TBS). Our studies show that this membrane can be used to anchor hexa-histidine-tagged molecules in a way that allows them to diffuse laterally and interact, thereby mimicking the cell surface.

EXAMPLE 1

The IAsys biosensor can be used to study the interaction of hexa-histidine-tagged proteins anchored onto the lipid bilayer.

A planar bilayer membrane consisting of PC and PE-NTA was formed on the sensing surface of the IAsys biosensor cuvette. Figure 2(a) shows the refractive index change due to formation of the bilayer (indicated Memb); there is no signal following two lots of washings with TBS (as marked), indicating that a stable bilayer is formed. Subsequently, the biosensor showed no change in refractive index on addition of BSA (see Fig. 2b), indicating a low level of non-specific binding. However, the addition of a 13 kDa transcription factor with hexa-histidine tag (denoted by HH in Fig. 2b) resulted in a strong signal indicating binding of this protein to the NTA-PE in the membrane via the NTA chelating linkage. The anchored protein was not removed after washing and incubating in TBS, but was removed with 200 mM imidazole known to break the chelating linkage (denoted Imid+TBS). Moreover, the interaction of other molecules with this protein also could be readily detected (not shown). The results show that hexa-histidine-tagged proteins can be stably and effectively anchored onto the bilayer to study molecular interactions with the biosensor.

EXAMPLE 2

Molecules anchored onto the lipid bilayer can diffuse laterally and interact.

A microscope slide was reacted with octadecyltrichlorosilane to form an octadecane monolayer (as described above). A phospholipid layer was then formed on the monolayer by placing a drop of the micelle (e.g. liposome) suspension (PC and biot-PE, 10:1) and incubating for 2 hours. After washing, the surface of the bilayer was stained with fluoresceinated streptavidin, incubated with Biot-BSA at 4°C, and then examined for fluorescein fluorescence. Preliminary studies show that under these conditions the fluorescence is seen uniformly over the surface. However, after a 30-60 minute incubation of the slide at 37°C the fluorescence is seen in discrete patches, indicating that the fluoresceinated streptavidin forms aggregates with the biot-BSA. This shows

that, depending on the temperature, molecules on the bilayer can diffuse laterally and interact.

EXAMPLE 3

Using the technique of this invention to resolve current difficulties in understanding of the CD4-MHC Class II interaction.

One application of the invention described herein is to study the interaction of CD4 with Major Histocompatibility Complex (MHC) Class II molecules, two key molecules involved in initiating an immune response by white blood cells. Crucial for the initiation of an antigen-specific immune response is the interaction of CD4 with invariant regions on the MHC Class II molecule, which provides adhesion and stabilizes the interaction between the T cell receptor and the presented antigen. Although cellular immunology experiments strongly suggest that such an interaction is crucial for efficient T cell activation and the triggering of an immune response, to date no direct evidence of this interaction or how it takes place, has been obtained.

Recent X-ray crystallographic studies on the three-dimensional structure of the extracellular portion of the human MHC Class II (HLA-DR1) molecule have shown that this molecule crystallizes as a dimer of noncovalently associated heterodimers. This suggests a capacity of the MHC Class II to dimerize, perhaps leading to an increased avidity for the CD4 receptor. The crystal structure of human soluble CD4 has shown that the D1-D2 and the D3-D4 regions of CD4 crystallize in multimeric form; but the affinity of self-association must be low because multimers do not form in solution. It has been suggested, that the extreme concentration of soluble CD4 in the crystal may reveal a natural propensity for oligomerization. Interestingly, biochemical evidence indicates that some CD4 immunoprecipitates as a disulfide-linked homodimer from lysates of sheep lymphocytes. Recently, evidence that human CD4 can oligomerize also was obtained using CD4 chimeras in transfectants, and by chemically cross-linking adjacent CD4 molecules on the cell surface. However, these findings do not clarify whether oligomerization is essential to increase the avidity of the CD4-MHC Class II interaction. Application of the technique as described below will provide the means to redress this deficiency.

EXAMPLE 4

The aim of this example is to determine whether the extracellular regions of mouse and human CD4 can oligomerize when anchored onto bilayer membranes that allow lateral CD4 mobility.

The atomic force microscope can be used to study the oligomerization of CD4 when anchored onto a lipid bilayer. A phospholipid bilayer consisting of 90% PC and 10% PE-NTA can be formed on a freshly cleaved and atomically flat surface of mica to permit high resolution atomic force microscopy. The procedure for this is the same as that used for forming bilayer on the biosensor surface (see above). Alternatively, the surfaces of glass-like materials, or surfaces coated with a uniform layer of gold (400-1000 A thick) using sputtered techniques, can be suitably treated with compounds like octadecyltrichlorosilane and octadecanethiol, respectively, to form a hydrophobic monolayer of hydrocarbon chains covalently attached to the surface and capable of reacting with a suspension of micelles (e.g. liposomes) to produce a hybrid bilayer consisting of the attached monolayer of octadecane hydrocarbon chains and the phospholipid layer comprised of PC/PE-NTA.

The recombinant extracellular region of CD4 with hexa-histidine tail can be anchored using the NTA chelating linkage onto bilayers formed on the glass or mica surface suitable for use in the atomic force microscope. The particular PC used to produce the PC/PE-NTA bilayer membrane can be chosen with chain lengths such that the membrane transition temperature is about 23°C (eg. dimyristoylphosphatidylcholine). Atomic force measurements can then initially be carried out with the system maintained at 10°C to prevent CD4 lateral mobility to map the force structure with CD4 in the monomeric state. The temperature can then be increased to 37°C for 30-60 minutes to allow CD4 to diffuse and interact laterally, before again cooling the system to 10°C to map the structure. Comparison of three-dimensional force maps of the CD4 molecules under the different conditions should allow determination of whether murine CD4 can oligomerize.

The IAsys biosensor can be used to measure the binding affinities (association and dissociation constants) for the CD4-MHC Class II interaction at 10°C and 37°C, using CD4 anchored onto

bilayers formed on the sensing surface of the biosensor cuvette. The finding of a higher binding affinity for the interaction of CD4 with soluble MHC Class II after subjecting to temperatures above the membrane transition temperature would provide further evidence that the extracellular region of CD4 has oligomerized. These experiments initially can be carried out using murine CD4 and MHC Class II with and without bound peptide. Similar experiments can be carried out using anchored human CD4 and soluble human MHC Class II. The force structure of CD4 anchored onto bilayers under conditions expected to exist in oligomeric form also can be mapped after binding soluble MHC Class II, if the interaction is stable to permit analysis, to see if MHC Class II binding can induce changes in the structure.

EXAMPLE 5

The aim of this example is to determine whether the extracellular regions of mouse and human MHC Class II oligomerize when anchored onto bilayers that allow lateral MHC Class II mobility.

Analogous to the studies described above for CD4, the atomic force structure of murine MHC Class II when anchored onto the bilayers can be mapped with the system maintained at 10°C, and compared with that observed after exposure to 37°C, to see if oligomerization occurs. The studies also can be carried out with and without peptide bound to the MHC Class II groove (for the mouse system), to determine if bound peptide has an effect on MHC Class II oligomerization. The atomic force structure also can be mapped after binding soluble murine CD4 to see if this can induce changes in the force structure and/or promote MHC Class II oligomerization. The biosensor can be used with membrane-anchored MHC Class II to measure the binding affinities of the interaction with soluble CD4, under conditions in which the MHC Class II is expected to exist in either monomeric or oligomeric form. The finding of a higher binding affinity of the MHC Class II for soluble CD4 after subjecting the system to temperatures above the membrane melting temperature, may provide additional evidence that MHC Class II can oligomerize.

EXAMPLE 6

The aim of this example is to test the hypothesis that oligomerization of CD4, and oligomerization of MHC Class II, are both required for CD4-MHC Class II interaction.

Failure to detect binding of either monomeric or oligomeric CD4 to soluble MHC Class II, and either monomeric or oligomeric MHC Class II to soluble CD4 in the experiments outlined above could indicate that oligomerization of CD4 and MHC Class II are both necessary for stable interaction. If both CD4 and MHC Class II can be shown to oligomerize in the membrane system, this possibility can be tested by examining whether CD4 and MHC Class II are able to interact under conditions where both are expected to exist in oligomeric form. This can be done by anchoring hexa-histidine-tagged MHC Class II onto unilamellar vesicles using the NTA chelating linkage, expose the vesicles to 37°C to permit MHC Class II oligomerization, and study the ability of these vesicles to interact with oligomeric CD4 using the IAsys biosensor. This can be done in both the mouse and human systems, but since the mouse MHC Class II with and without peptide in the groove is available, the mouse MHC in the presence of bound peptide may be required to achieve the correct molecular confirmation required for the interaction.

The feasibility of this approach has been demonstrated recently by studies showing the interaction of biotin-PE and PC-containing vesicles with strepavidin anchored onto a lipid bilayer formed on the IAsys biosensor surface. Figure 3 is a biosensor trace showing changes in refractive index due to additions made to an IAsys biosensor cuvette after reacting the sensing surface of the cuvette with a suspension of vesicles consisting of PC and biot-PE. The initial change in refractive index is due to the formation of the bilayer. Subsequently, the cuvette was washed with Tris buffer. After equilibration, the addition of BSA (indicated BSA) resulted in no change in refractive index, indicating a low level of non-specific binding. The addition of streptavidin (indicated Strep) resulted in a change in refractive index, which did not alter upon washing with Tris buffer (indicated Tris). The addition of a suspension of vesicles made of PC (indicated PC) did not bind to the membrane, but the addition of vesicles made of PC and biot-PC did interact with the membrane, reflecting the binding of the vesicles containing the biot-PE with the streptavidin anchored onto the membrane.

EXAMPLE 7

Using the instant invention to anchor or graft hexa-histidine tagged molecules onto the surfaces of cells and other biological and synthetic membranes (see Figure 4), for the development of vaccines and for drug targeting.

The histograms in Figure 5 show fluorescence-activated cell sorting (FACS) profiles of murine mastocytoma P815 cells carrying grafted recombinant hexa-histidine-tagged murine B7.1 and CD40. P815 cells were pre-incubated for 30 min at 37°C with a suspension (0.1 mM) of control lipid di-myristoyl-phosphatidylcholine (di-C14-PC, control), or the chelator lipid di-C14-NTA, before being washed in PBS and incubated with a mixture of hexa-histidine-tagged B7.1 and CD40 (each at ~20 mg/ml). The cells were then washed again in PBS and stained by an incubation (30 min at 4°C) with either biotinylated 16-10A1 or biotinylated B-3/23 monoclonal antibody (ie. biotinylated anti-B7.1 or anti-CD40), as indicated, followed by an incubation with FITC-conjugated streptavidin. Cells incubated with di-C14-PC and recombinant proteins show a low level of fluorescence after staining with either monoclonal antibody (Control). The fluorescence of P815 cells pre-incubated with di-C14-NTA is 10-100-fold higher than that of cells pre-incubated with di-C14-PC (Control). Each result is a representative of two experiments performed in duplicate. The results show that chelator lipids (in this instance di-C14-NTA) can be incorporated into the membrane of these cells, and that the incorporated lipid can be used to anchor or graft hexa-histidine tagged B7.1 and CD40 directly onto the P815 cell surface via the di-C14-NTA. In other studies we showed that recombinant murine B7.1 and CD40 bearing a hexa-histidine tag can be grafted onto the surface of all the different cell lines tested; these included murine P815 and EL4 tumor cells, human leukemic Jurkat cells and yeast cells.

EXAMPLE 8

The Example relates to modifying the surface of tumor cells to enhance tumor immunity.

Recent work indicates that the transmembrane and cytoplasmic regions of B7-1 and B7-2 are not required for T cell costimulation (23), and that T cell costimulation also occurs when the B7-1

is expressed on tumor cell surfaces in a GPI-anchored form (24). Also, the extracellular regions of any cell surface receptor molecules (eg. the murine T cell costimulator molecules B7.1 and CD40) can be produced to contain a hexa-histidine or other appropriate peptide tag on the carboxyl terminal. In this form the present invention provides a method of anchoring these costimulator molecules directly onto the cell surface in the correct orientation, thereby mimicking the costimulatory function of these molecules on the surface of antigen presenting cells. The instant invention, therefore, has implications for tumor vaccine development, by providing a more convenient and safe alternative to transfection for putting costimulator and/or other relevant molecules onto tumor cells for use in immunisations to enhance immunity to tumors.

The viability of using grafted molecules can be tested by assaying for functional responses dependent on the grafted molecules. Thus, the ability of murine P815 mastocytoma (DBA/2, H-2d) cells carrying grafted hexa-histidine tagged B7-1 and/or CD40 to stimulate a T cell proliferative response in an allogenic system was examined using splenocytes isolated from C57Bl/6 (H-2b) mice co-cultured with an appropriate number of g-irradiated P815 cells (as control), or P815 cells grafted with hexa-histidine-tagged B7-1 and/or CD40. Preliminary experiments in which the incorporation of 3H-thymidine was used to measure T cell proliferation (see Table 1), show that the P815 cells bearing grafted hexa-histidine tagged B7-1 and/or CD40 are able to stimulate an increased level of T cell proliferation in this mixed cell reaction. These results are consistent with the invention being useful to modify cells for use in vaccinations to enhance anti-tumor responses.

EXAMPLE 9

Since the Examples above use recombinant forms of the extracellular regions of receptor molecules oligomerization would be detected only if it arises from interactions via the extracellular region of the molecules. For many receptors this is unlikely to present difficulties as both the transmembrane and intracellular regions are short and are unlikely to be involved in oligomerization. Certainly, for the CD4 and MHC Class II described above, this notion also is supported by X-ray crystallographic studies which show that the extracellular domains of the molecules each can dimerize/oligomerize spontaneously in the absence of other molecules (e.g.

those in the cytoplasm). However, other applications of the technique also include the anchoring of the cytoplasmic or transmembrane domain of receptor molecules onto the model membranes to study their involvement in oligomerization and/or in interactions with other membrane associated or cytoplasmic molecules involved in signal transduction.

Another useful application of the instant technology is to study the interaction of CD2 with its ligands. Thus, the use of multimeric binding techniques have identified the glycosyl-phosphatidylinositol (GPI) anchored molecule CD59 as an alternative ligand for human CD2, but biosensor studies have failed to confirm the interaction of CD2 with monomeric soluble CD59. This indicates that this interaction is of low affinity and that oligomerization of CD59 may be required to form a stable interaction with CD2. Clearly, analogous to the technique used above for studying CD4-MHC interactions, the anchoring of CD59 onto lipid bilayers coupled with biosensor and atomic force analysis could be extremely useful in determining whether oligomerization of CD59 is required for interaction with CD2. This technique also is particularly suited in applications for studying the interactions of other GPI-anchored receptor molecules. Such receptor molecules could be anchored to the membrane either directly through their native GPI anchor, or by engineering a hexa-histidine tag to replace the native GPI moiety, and using the hexa-histidine-NTA chelating linkage to anchor the receptors onto the membrane.

The Examples described above clearly establish that the technique in conjunction with the technologies outlined is very useful for the analysis of other low affinity molecular interactions. The subject technology can also be used to assay for interactions or aggregation events between receptor domains, proteins, glycoproteins, polysaccharides, or fragments thereof, after attachment of an appropriate hexa-histidine tag and the anchoring of the molecules onto the bilayer membrane system. This provides a method of screening for agents or drugs that may influence such receptor interactions. The invention therefore, provides the basis of a novel method of screening for drugs or other agents that influence receptor interactions, and consequently, the ability of the receptors to transduce transmembrane signals and/or to elicit responses in biological systems.

EXAMPLE 10

The present invention may also be used to anchor any molecular species, or combination of different molecular species, possessing a suitable hexa-histidine or other peptide tag which can interact with the metal chelating headgroup of the chelator lipid incorporated into any cell type, subcellular membraneous component, or synthetic vesicles or liposomes. The instant invention provides a convenient and safe alternative to transfection for modifying cell surfaces, thereby facilitating the development of cell based vaccines, and vaccines involving the use of biological and/or synthetic membranous components. Such vaccines can be used clinically in patients whose conditions require specific alteration in aspects of immune function (e.g. to enhance their immunity to a specific tumor or disease). In addition, the present invention can be used in conjunction with liposomes for the targeting of drugs and other therapeutic agents to specific cells and tissues.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 1

Stimulation of T cell proliferation by B7-1/CD40 grafted P815 cells. Allogeneic T cells (1 x 10^5 T cells isolated from the spleen of Black/6 mice) were co-incubated with 1 x 10^5 γ-irradiated (5,000 Rad) stimulator cells; these included, P815 cells, di-C14-NTA-P815 cells, B7.1-diC14-NTA P815 cells or B7.1-CD40-NTA-di-C14-NTA P815 cells. After 48 hrs of co-culture at 37°C in complete growth medium the cells were pulsed with 1 μCi of [^{3}H]-thymidine for 16 hrs; the cells were then harvested and the level of [^{3}H-thymidine incorporation assessed.

Condition	^{3}H-thymidine incorporated (CPM)
T cells	637
P815 cells	514
NTA-P815	566
BSA-NTA-P815	996
B7.1-NTA-P815	1585
CD40-NTA-P815	1373
B7.1+CD40-NTA-P815	1410

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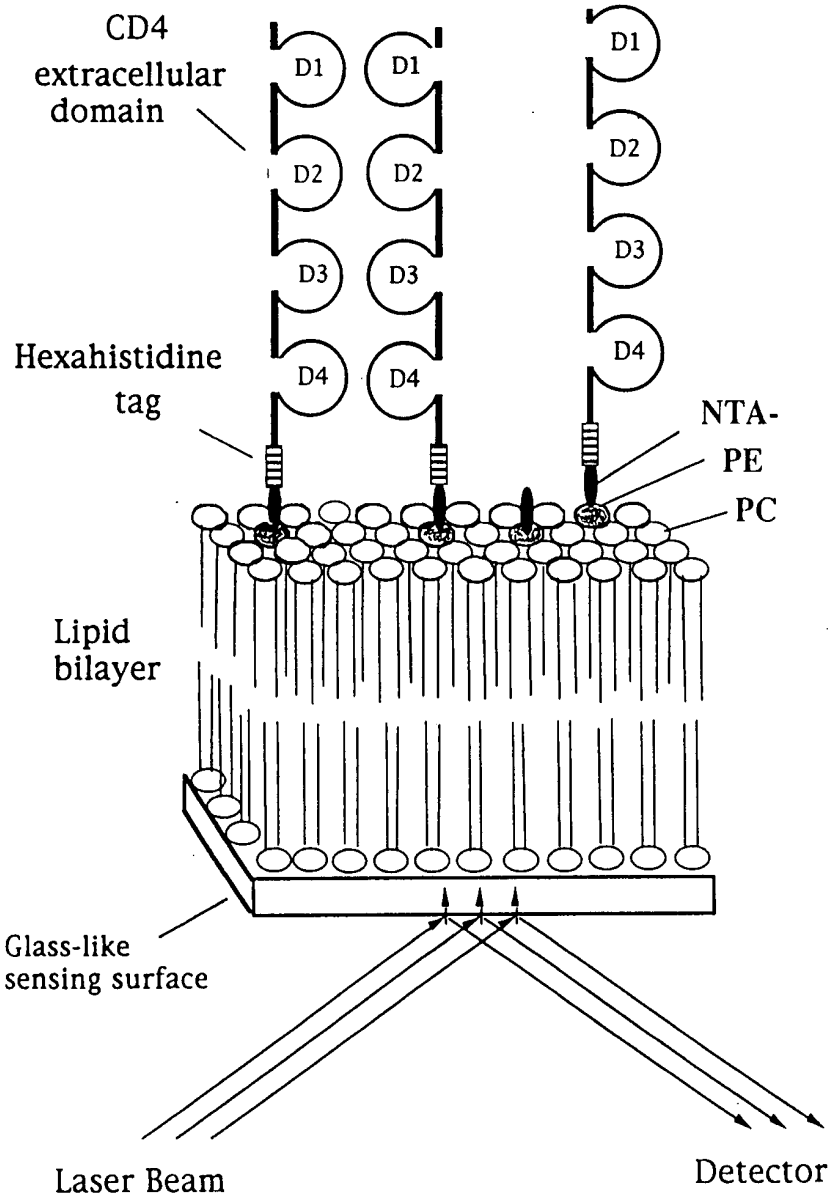
DATED this 28th day of April 1999

The Australian National University
by their Patent Attorneys
DAVIES COLLISON CAVE

Fig. 1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D2
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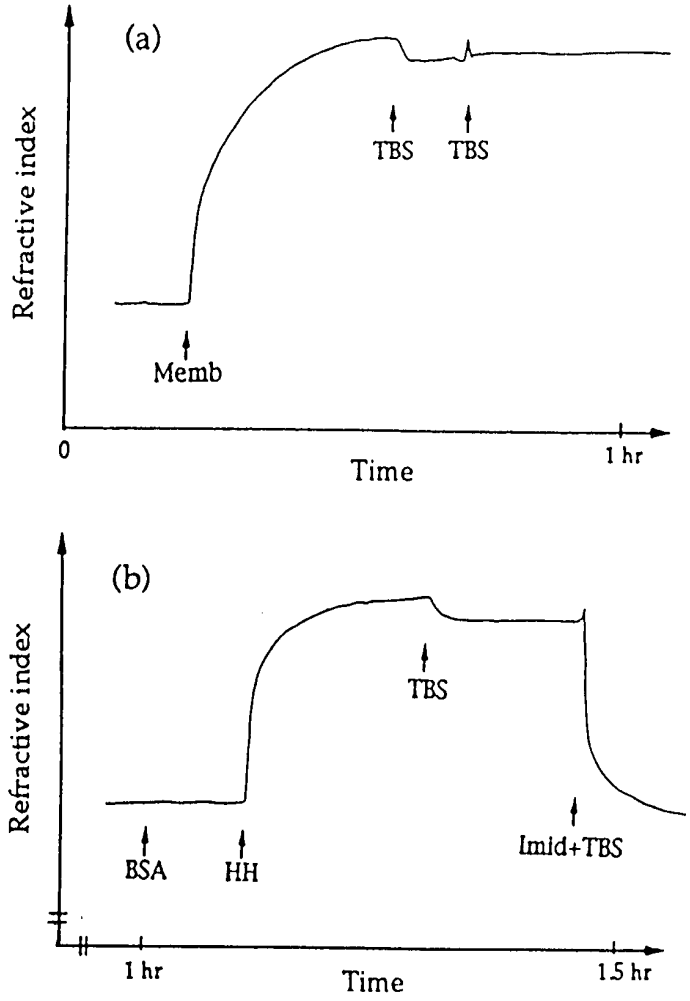
Laser Beam
Detector

Fig 2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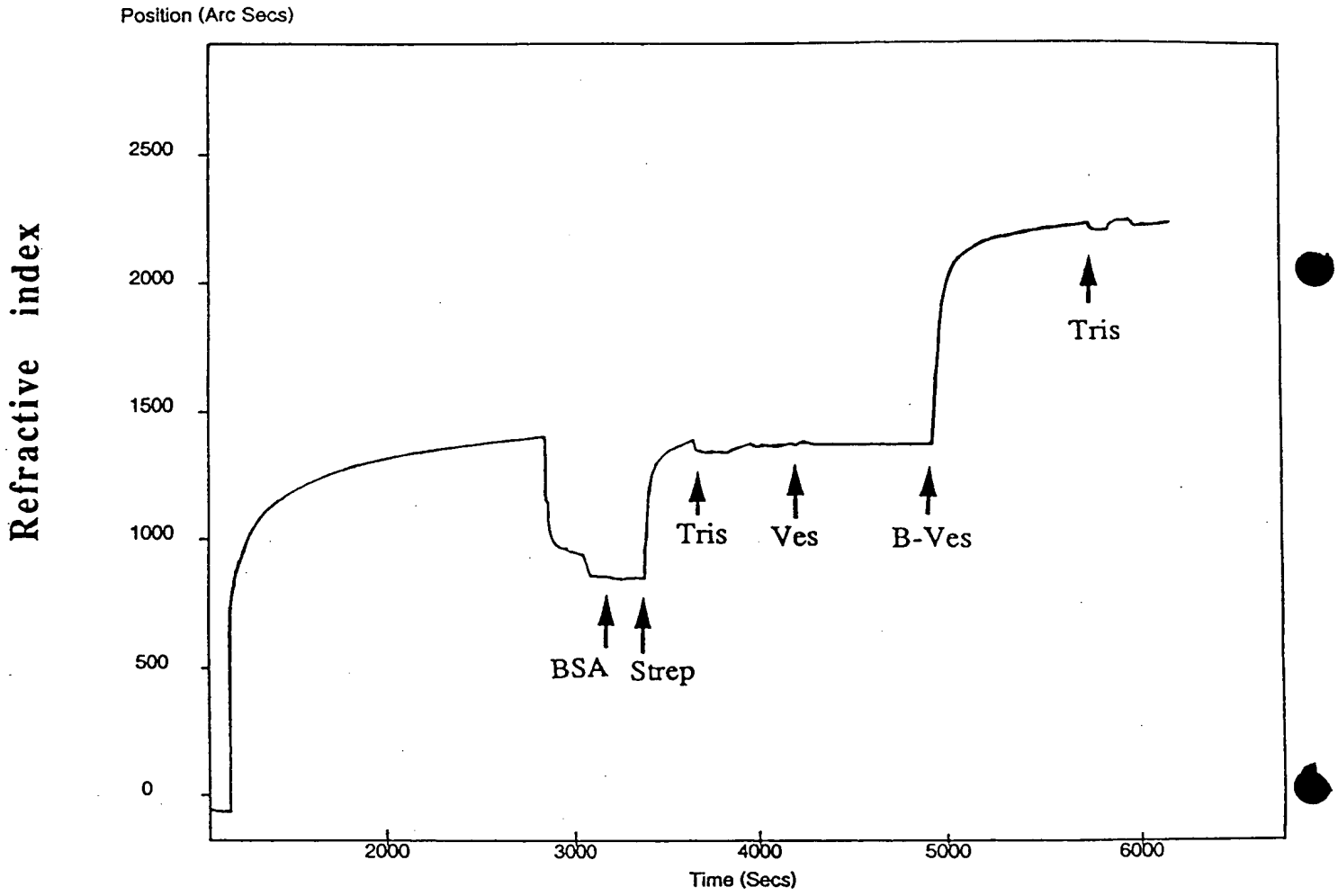
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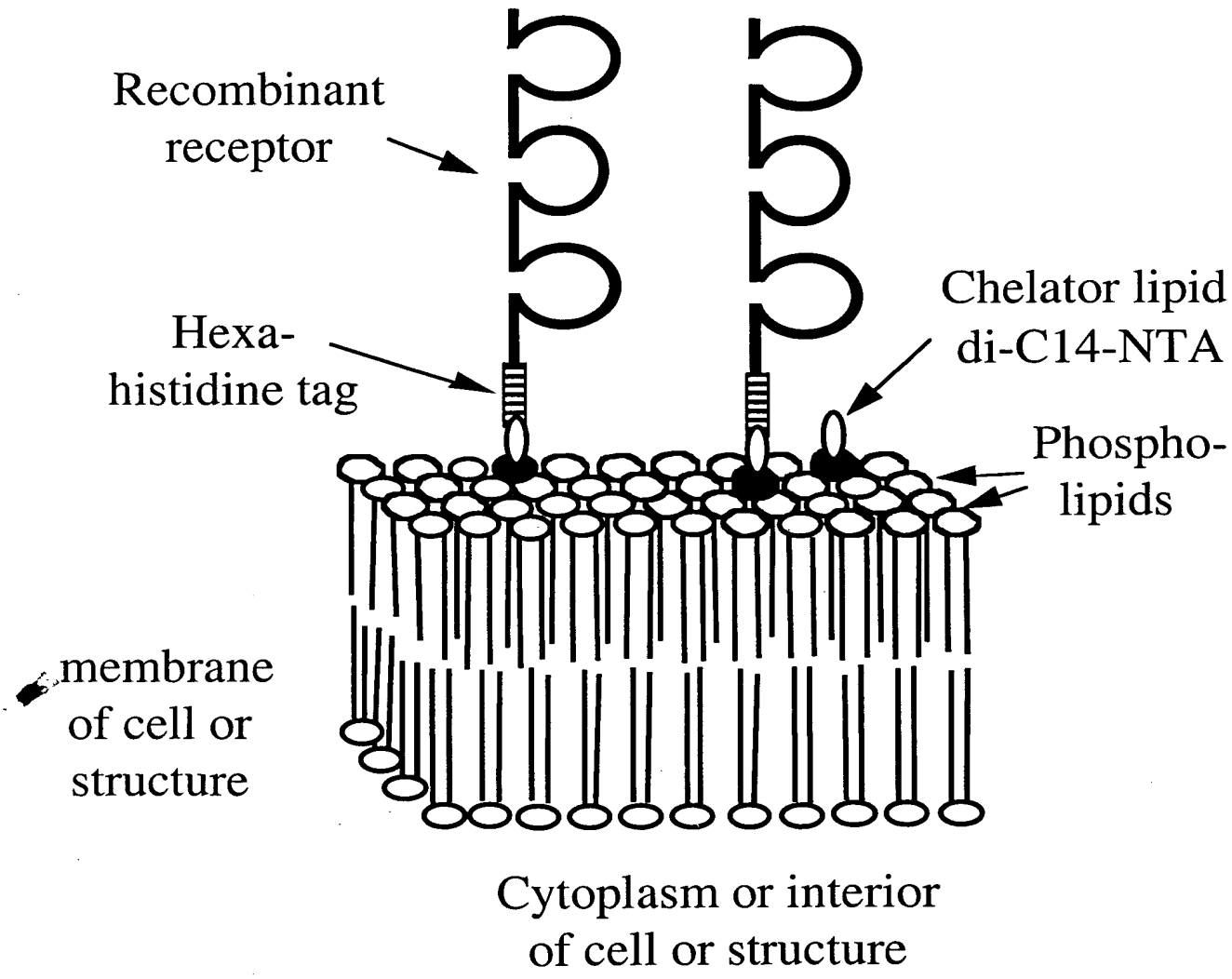
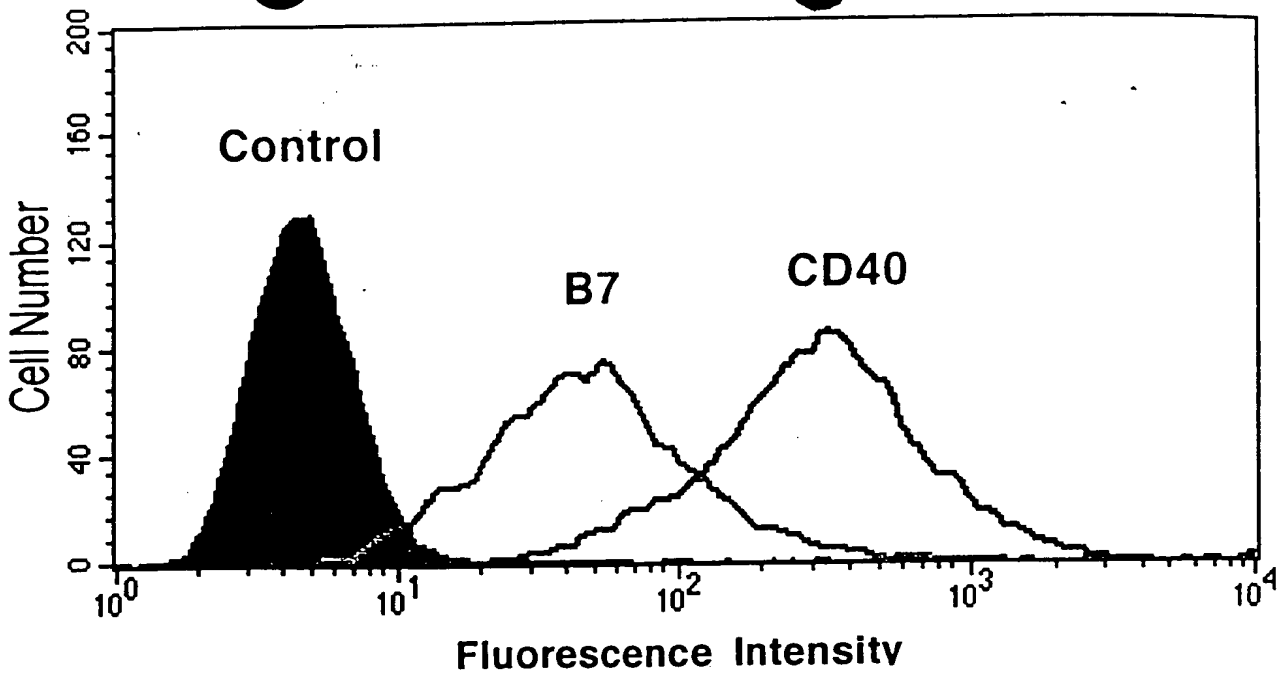
Fig 3

Figure 4.



The diagram depicts how recombinant receptors bearing a hexa-histidine tag can be grafted onto biological membranes such as the plasma membrane of cells or the membrane of subcellular membranous structures, and onto the surface of artificial vesicles or liposomes. The recombinant protein is grafted onto the membrane structure through the binding of the hexa-histidine tag on the protein to the NTA metal chelating headgroup on the chelator lipid (denoted di-C14-NTA) which has been incorporated into the phospholipid membrane.

Fig. 5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200
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10^1
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10^4
Fluorescence Intensity